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GENETIC STUDIES WITH BACTERIA

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GENETIC RECOMBINATION

GENETIC investigation is by no means confined to breeding experiments, nor even to organisms exhibiting a sexual stage which makes such experiments possible. But recombination provides such a powerful tool for the analysis of the genotype as to be almost a *sine qua non* for decisive research. Of the three main criteria for the dissection of the genotype—physiological effect, mutational relationships, and recombinational separation—the last is at once the most utilitarian and the most delicate.

Bacteria, for obvious reasons, provide exceptionally favorable material for physiological research. Fortunately, it is no longer necessary to deplore their apparent exclusively asexual reproduction as a limitation to genetic applications. In at least one bacterium, *Escherichia coli* (the common colon bacterium), genetic recombination can be shown to occur. In addition to providing a sound basis for phenogenetic research in bacteria, the demonstration of genetic reassortment necessitates a search for the presumably sexual morphological processes which can be inferred from it.

We may suspect that if recombination occurred frequently, it would have been detected and confirmed long since. It is, therefore, desirable to design a selective method for the screening of large populations of bacteria for genetic recombinants. Such a method was applied by Tatum and myself at Yale University in 1946 with the help of nutritional mutants of *E. coli*, strain K-12 (Tatum, 1945).

A nutritional mutant is a culture which has become *auxotrophic*, that is, dependent upon an external source of some metabolite for its growth. It differs from the nutritional wild type, or *prototroph*, by its inability to effect the synthesis of some essential protoplasmic constituent which the prototroph can manufacture from simple compounds. Wild type or prototrophic *E. coli* cells can synthesize all needed metabolites from glucose and inorganic salts, and therefore grow well in such a medium. Nutritional mutants, on the other hand, require that the medium be supplemented with their specific growth factor requirement, which may be a vitamin such as biotin, an amino acid like methionine, or purine or other compound, as the case may be. Nutritional mutants of *E. coli* are essentially similar to those first produced in Neurospora by Beadle and Tatum (1941, 1945) a decade ago. However, technical advances permit much more efficient and direct recovery of bacterial mutants than is possible for filamentous fungi (Davis, 1950a; Lederberg, 1950c).

As shown in Figure 1, plating mixtures of auxotrophs into the minimal glucose-salts medium permits the selective isolation even of infrequent prototrophic components (Tatum and Lederberg, 1947). Since prototrophs form a set of the recombinants to be expected from genetic exchanges between two different mutants, the plating of such mixtures on minimal agar constitutes a sensitive test for genetical recombination. Precautions are needed, of course, to ensure that these prototrophs do not arise merely by reverse mutation of one of the parents. This complication is most readily disposed of by using multiple, rather than single, mutants as parents.

The prototrophs provide the putatively sexual progeny which can be surveyed for factor recombination. It must be emphasized that the demonstration of recombination does not rest directly upon the mere observation of prototrophs, but upon the occurrence of a series of new combinations of unselected markers introduced with the nutritionally differentiated parents. In Figure 2, B, M, T, L represent nutritional factors (biotin, methionine, threonine, and leucine) which are used for the selective isolation of prototroph recombinants. B_1, Lac, and V represent factors for thiamin-requirement, lactose fermentation, and virus-resistance. In a glucose-synthetic medium to which thiamin is added, these markers are unselected, and should be

free to assort in any combination, parental or new. Prototrophs occur in such a "cross" with a frequency of about one per million cells inoculated. All of the possible combinations of the unselected markers, as listed, have been found among the prototrophs, although not with equal frequency, suggesting mechanical limitations on re-

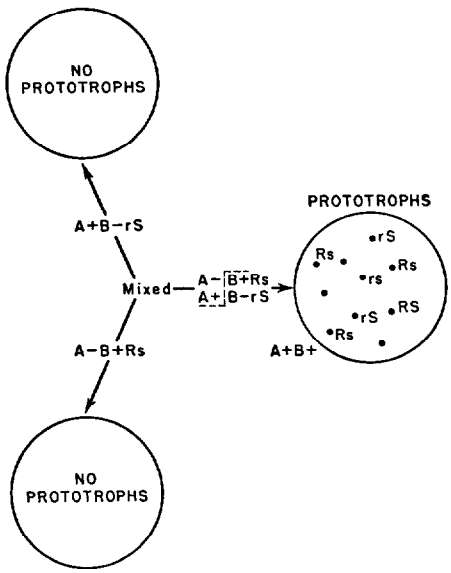

FIG. 1. Selection of recombinant prototrophs from mixtures of nutritional mutants. A and B refer to nutritional factors; r and s to unselected markers the alternatives of which are free to reassort among the prototrophs. The large circles represent plates of minimal agar medium.

combination (Lederberg, 1947). The same type of result has been obtained with great regularity with many other selected and unselected markers, and in the hands of workers in a dozen different laboratories. About forty mutant characters have been used in these experiments, each conforming to this general behavior.

The prototrophs produced in these experiments are ordinarily pure and stable from the time that the prototroph colony is initiated,

although, of course, different prototrophs may carry different combinations of unselected markers. Segregation thus occurs prior to the growth of the recombinant prototroph. From this it follows that the typical vegetative form is haploid, and that the diploid "zygotic" stage is transient, and undergoes immediate segregation, associated with recombination which sometimes produces a detectable prototroph.

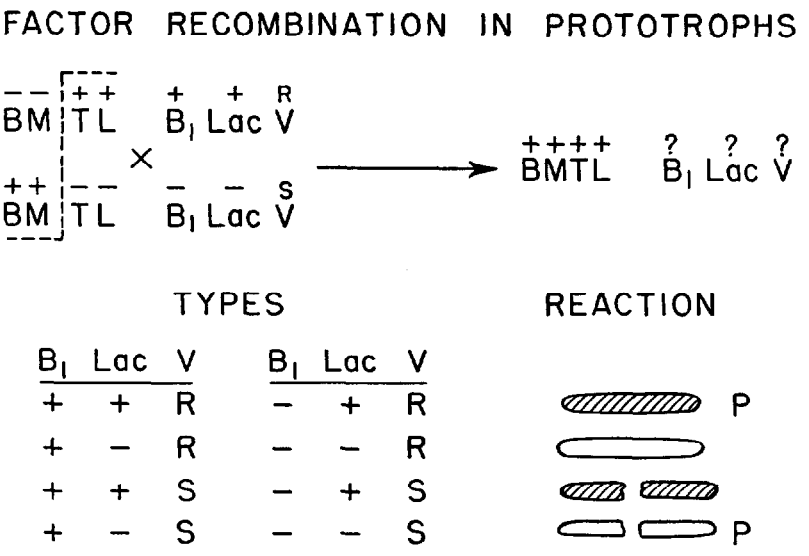

FIG. 2. Diagrammatic representation of a cross in which B_1, Lac, and V are unselected markers. All eight of possible recombinations of these factors are found among the prototrophs. The phenotypic appearance of various combinations with respect to Lac and V is shown at the lower right (test carried out by cross-streaking culture with virus on lactose-indicator-medium).

Since zygote formation has not yet been directly observed, owing to its relative rarity, the evidence for its occurrence is largely based upon indirect genetic inference. However, the efforts of several workers besides ourselves to accomplish the genetic transfer with nonliving products of parental cells have been entirely fruitless. Davis (1950b) has shown that the interposition of a sintered glass filter between potentially interacting cultures completely prevents recombi-

nation, although the culture medium be forced repeatedly from one chamber to the other. This result suggests that the gametic unit is of the same dimensions as the bacterial cell. Other genetic experiments have shown that the gametic unit must likewise be large enough to encompass most or all of the genotype since so many factors can be exchanged at once. Experiments involving mixtures of three well-marked parental types have shown further that recombination occurs only between single pairs of genotypes, that is, that the gametic units remain intact and do not mix with each other prior to the formation of the putative "zygote."

Before the statistical distribution of markers among prototrophs can be interpreted, it is necessary to verify that this distribution is based upon the mechanical properties of the system, rather than upon the physiological effects of the markers. This can be done in "reverse" crosses, in which a mutant marker is introduced first with one parent, and then the other. With a variety of markers, the segregation of two alleles among prototrophs is quantitatively inverted when the alleles are introduced in reverse combinations. This verifies the concept of the haploid life cycle, and also supports the use of these mutants as indifferent markers or tags for the corresponding parts of the genotype throughout the mechanics of recombination (Lederberg, 1947).

By the nature of these crosses, it is difficult to map these markers, and simultaneously to prove their linear arrangement in linkage without a certain element of logical circularity. However, a number of workers, including Newcombe and Rothfels, L. Cavalli, Gordon Allen and myself, have been able to show with reasonable certainty that many factors can be placed in an unambiguous linear order, although in some instances a sufficient excess of multiple-crossover types has been found that Newcombe has suggested the presence of a certain degree of negative crossover-interference. Unfortunately, however, there is, as we shall see, some suspicion that the segregation behavior of single markers in these crosses may be more complicated than has hitherto been thought, so that detailed linkage interpretations should perhaps be postponed until the behavior of single factors is further clarified.

The analysis of linkage is, of course, greatly impeded by the fact that only a particular recombination subset, the prototrophs, is available with this technique. It would certainly be preferable to have strains or conditions in which zygote formation occurred frequently enough that the selective method could be dispensed with. This has not yet been accomplished, although Cavalli (personal communication) has found unusually "fertile" strains, which recombine perhaps a hundred times more frequently than type. In a related study, T. C. Nelson working at Columbia University, has examined the kinetics of prototroph formation as a function of parent concentration and the time of their contact. His results, which show that the initial reaction between parents can be treated kinetically as a "bi-particulate" reaction, should place on a sound base the study of recombination rates as a function of genetic constitution and environmental conditions.

Prototrophs are not the only recombinant types which can be selected. Gordon Allen has demonstrated the feasibility of searching for complementary genotypes by nutritional selection of recombinants on synthetic medium to which certain supplements are added, followed by the plating of such recombinant colonies into medium with the complementary supplements.

It is also possible to dispense with nutritional selection altogether, by preparing two mutants each resistant to one inhibitor such as streptomycin and sodium azide, respectively, and plating mixed cultures into a medium containing both inhibitors. Dually resistant recombinants can be distinguished from less frequent spontaneous mutants by the recombination of other, unselected markers (Lederberg, 1950a).

NON-DISJUNCTIONAL EXCEPTIONS

A more accurate, if not a clearer picture of segregation mechanisms has been obtained from the behavior of certain non-disjunctional exceptions, or diploid hybrids (Fig. 3). Certain stocks have been found in which segregation is delayed, so that a large proportion of isolated prototrophs continues to segregate marker differences introduced with the parents (Lederberg, 1949). This is in distinction to

the typical behavior of prototrophs, mentioned earlier, which are already segregated and genetically pure. The exceptional prototrophs are unstable during culture, and segregation occurs from time to time, with a frequency of the order of once per twenty fissions (Zelle and Lederberg, unpublished). Thus, exceptions heterozygous for lactose fermentation (Lac+/Lac−) form variegated colonies

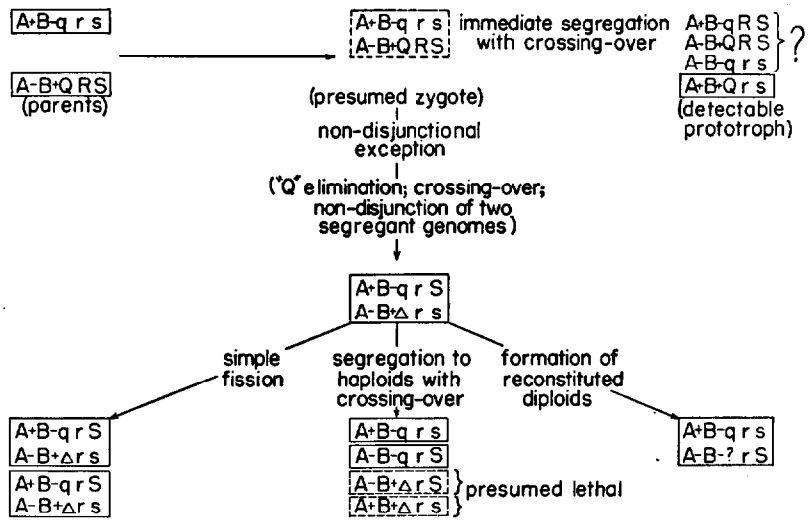

FIG. 3. Inferred pathways of segregation of normal and non-disjunctional hybrids. A and B refer to nutritional characters; q, r and s to unselected markers. Q includes those markers (Mal;S) which are frequently eliminated from the diploid cells. The elimination leads to a deficiency symbolized with a *delta*.

on lactose indicator medium, the colonies consisting of cells still heterozygous in addition to apparently haploid, genetically pure Lac− and Lac+ segregant types (Fig. 4).

If these exceptions segregated solely for the Lac+/− character, one might suspect that the variegation was due simply to mutational instability at one locus. Indeed, a unique example of such an unstable gene, for maltose fermentation, has recently been found. However, segregation of Lac is invariably accompanied by segregation for the remainder of the hybrid genotype, so that the process must be

thought of as the separation of two genomes. That such genomes are indisputably contained within single bacterial cells has been shown by elaborate single-cell pedigree studies by M. R. Zelle.

The segregation usually results in haploids which carry one or the other parental combination of markers, so that each of the genomes of the hybrid tends to preserve its integrity. However, recombinant segregants also occur, with varying frequency depending upon the stock and the markers considered. This excludes the possibility that the exceptions are simple heterokaryons. All of these facts are consistent with the simplest analogy which we can cite, that the exceptions are diploid heterozygotes entirely comparable to those of higher

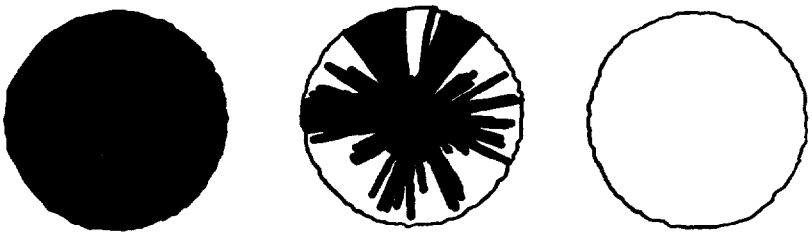

FIG. 4. Appearance of heterozygous and haploid colonies on indicator agar. The mosaic appearance of the heterozygote is due to frequent segregations which unmask the recessive Lac—.

plants and animals. A more detailed analysis has shown, however, that these exceptions cannot be regarded simply as unreduced zygotes, but that a rather complex history of crossing-over, segregation, non-disjunction, and segmental elimination must be postulated for the exceptions if they are to be interpreted in terms of the chromosome mechanics already familiar to us.

The first anomaly consists in the fact that the unreduced exceptions, selected as prototrophs variegated for lactose, xylose, mannitol, or galactose fermentation, are regularly pure for maltose fermentation. About 95 per cent of the exceptions carry only the Mal allele contributed by the T−L−B_1− parent; the other 5 per cent carry the alternative allele, from the B−M− parent. Thus the majority of the unreduced prototrophs from a particular cross appear to be

(B−M− T+L+B_1+//B+M+ T−L−B_1−; Lac+ Xyl+ . . . // Lac− Xyl− . . . ; but Mal−). The question arises whether the observed purity for Mal reflects hemi- or homo-zygosity for this locus, that is, one or two representations of this gene. This has been tested by observing spontaneous reverse mutations from Mal− to Mal+, which occur infrequently (ca. 10^{-7} per fission) but detectably. Such reversions have invariably resulted in stocks which became pure rather than segregating Mal+, pointing to the hemizygous condition of this locus. If so, one of the parental Mal genes must have been eliminated or deleted. That the deletion covers a finite segment is suggested by the finding that factors such as S^r/S^s (affecting resistance to streptomycin), linked to Mal have likewise never been recovered in heterozygous form. In Figure 3, this region is symbolized "Q."

A second type of anomaly has, perhaps, a simpler explanation. In contrast to the regularity with which Mal occurs pure in diploid exceptions, occasional exceptions occur which are heterozygous for some factors, but pure for Lac or for Xyl. Pure Lac− types have been tested for hemi- versus homo-zygosity by the study of Lac reversions from minus to plus. Here, one always finds that the pure Lac− type reverts to a variegated Lac+, pointing to homozygosity. That is, Lac−// Lac− gives rise to Lac−// Lac+. The consistently different results obtained when this test is applied to Mal-pure and Lac-pure exceptions help to support its validity as a test for hemi- and homozygosity. Homozygosity for factors in which the parents differ suggests that the exceptions are not the original zygotes, which should be uniformly heterozygous, but the result of non-disjunction of two products of crossing over. In this respect, they closely resemble cells of mosaic spots of heterozygous, Minute *Drosophila melanogaster* (Stern, 1936) except that in *E. coli* we have only the spots, and must infer the normal fly from them. On the same argument applied by Stern to somatic crossing-over in Drosophila, it may be inferred that crossing-over in *E. coli* involves a four-strand system.

A third anomaly concerns the aberration of monofactorial segregation ratios. Where an equal separation of segregants into two alternative classes is expected, ratios deviating as far as 15: 1 are found, the

magnitude of the deviation depending upon the particular factor. This aberration may, however, be regarded as a simple consequence of the elimination of the "Q"-region already discussed. If "Q" is a segment of a chromosome, other factors not eliminated but linked to it will not appear among viable segregants, unless crossing-over supervenes between "Q" and the factor. This presumes that half the segregant genomes are lost, owing to the haplo-lethal deficiency for "Q," represented in Figure 3 with a *delta*. Some support for this concept may be gleaned from Zelle's single-cell studies, for he finds a rather high frequency of inviable and morphologically peculiar cells in single-cell pedigrees of diploid cultures. It remains to be seen whether inviable cells are unique to diploid cultures which are presumably segregating a haplo-lethal deficiency, or whether they originate from irrelevant causes.

Still another pathway of genetic change has been found in an exception among the exceptions, an isolated instance (among several hundred tests) of a diploid heterozygous for Mal. This diploid most frequently follows the previously established modes of proliferation: simple fission or segregation to haploids. About one one-hundredth as frequently as it forms haploid segregants, however, it gives rise to reconstituted diploids in which some factors previously heterozygous become homozygous. Whether cells are produced which are uniformly homozygous is not known, but from the standard which is (Lac+/− Mal+/− Xyl+/− . . . .) reconstituted diploids have been found which are (Lac−/− Mal+/− Xyl+/−) or (Lac+/− Mal−/− Xyl−/−) and so forth. The designation of the Lac-pure and Mal-pure derived types as homozygous (not hemizygous) is based upon reverse-mutation tests like those previously discussed. Of particular interest is a study of reversions of a reconstituted Lac+/− Mal−/− Xyl+/−. Mal is quite closely linked to Xyl, so that this stock provides an opportunity to distinguish two kinds of reversion from Mal−/Mal− to the heterozygous condition, namely: Mal+ Xyl+/Mal−Xyl− and Mal+Xyl−/Mal−Xyl+. The Mal reversions of this stock have been found to fall into these two classes (coupling and repulsion) in approximately equal numbers. The result has little decision value, but it may help to support the

premise that we are dealing with heterozygous diploids whose genetic structure is basically comparable to that of higher organisms, although deviations such as segmental elimination interfere with an uncomplicated demonstration.

It is not clear whether immediate segregation yielding "normal" prototrophs follows a different and simpler course. That some or all of the same peculiarities may apply is suggested by a number of findings. Unreduced prototrophs occur most frequently in the progeny of special "*Het*" stocks (Lederberg, 1949a), but can also be detected in standard crosses with the help of two very closely linked Lac− factors in repulsion. Crossing $Lac_1- \times Lac_4-$ results in prototrophs most of which are lactose-negative. About one-tenth per cent, however, are Lac+. Most of these turn out to be unreduced $Lac_1- Lac_4+/Lac_1+Lac_4-$ diploids rather than Lac_1+Lac_4+ crossovers. Such Lac+ exceptions can be detected by carrying out crosses on a synthetic indicator medium. These exceptions from normal crosses have the same behavior in every particular as the more frequent exceptions from "*Het*" crosses. In addition, it has been found that the Mal factor which behaves so peculiarly in diploids cannot be placed on a linear linkage map with other factors in haploid prototroph segregations. Mr. Gordon Allen (personal communication) has obtained some preliminary evidence for the occurrence of complementary nutritional types from single zygotes, and these also show unorthodox behavior. Finally, I have recently found several occurrences of segregants (from diploid exceptions) which when used as parents in F-2 or backcrosses give entirely unique linkage relationships, although phenotypically identical with the original parent. These segregants must be regarded as structurally different from their phenotypically similar parent, but it cannot yet be said whether the segregant or the parent carries the standard gene arrangement. At any rate, until these questions are cleared up, it will be necessary to deal very cautiously with the problems of linkage in *Escherichia coli* K-12. For this reason, I will not attempt to insist upon the evidence for linear organization (Lederberg, 1947), although as far as it goes it appears to be entirely self-consistent.

A cytological comparison of haploid and diploid cells has been

initiated in this laboratory by Miss E. Lively. Distinctive differences in the texture and organization of the nuclear material have been found, which do not yet, however, admit of a clear interpretation, owing especially to the multinucleate condition of most rod-shaped bacterial cells.

BACTERICIDAL MECHANISMS

An application of diploid *E. coli* which may be of some interest is to the problem of the genetic mechanisms which are involved in killing by radiations and chemicals. Although it is not possible to offer a clear picture of the mechanisms which are involved, recessive lethal mutations play a negligible role in the killing of bacteria by ultra-violet light or by x-rays. This is shown by the rarity of balanced lethal types among the survivors of various doses of these radiations. Atwood (1950) has reached a similar conclusion from studies on the irradiation of heterokaryotic Neurospora conidia. Rather complex genetic changes are found following irradiation of heterozygous diploid bacteria, in particular haploid and reconstituted diploid cells. These changes can be detected in populations showing 90 to 95 per cent survival, so that there can be no question of selective survival. The widespread genetic reorganization may be correlated with the accumulation of nuclear material observed in "snake" cells found in populations recovering from radiation (Delaporte, 1949).

Similar genetic effects, that is, haploids and reconstituted diploids, have been observed in survivors of populations treated with such diverse reagents as nitrogen mustard, hydrogen peroxide, formaldehyde, acetic anhydride, dimethyl sulfate, and ethylene oxide. Although, in common with radiations, many of these compounds are known to be mutagens, and all may be, the relationship between genetic reorganization, sterilization, nuclear pathology, and mutation is not clear.

In some laboratories, bacterial cell suspensions have been thought of as naked genes with which to do experiments on chemical modification, that is, mutagenesis. This view is not acceptable without better evidence than is now available. Recent discoveries have tended to weaken the supposition that radiations produce their genetic effects

by direct interaction with genetic material. In much the same light, we must be very cautious in interpreting chemical mutagenesis as a direct chemical reaction with the gene. Cells, including bacteria, react in a very complex pattern to treatment with mutagenic agents. The possibility cannot be excluded that some mutations are produced indirectly as a consequence of accidents during recovery or of non-specific and non-localized disturbances of nuclear structure.

In view of the great diversity of reagents which have mutagenic or other nuclear effects, it is reassuring that some have been found which do not. In experiments with diploid *E. coli*, heat, iodine, and streptomycin were found to kill cells without any detectable genetic concomitants.

GENETIC ANALYSES IN ESCHERICHIA COLI K-12

At this point, I should like to take up a few examples of genetic analysis using recombination methods in order to illustrate the scope and technical facility of these approaches.

Reverse mutation

Mutations leading to the reassumption of the wild phenotype have been noted frequently in bacteriological studies (Ryan, 1946). In fact, the misconception that gene mutations in other forms were irreversible served, at one time, as an argument against the mutational basis of bacterial variations. Such reversions, however, must be studied by crossing techniques before it can be determined whether they represent reverse-mutations of the mutant gene, or changes of distinct loci or other determinants. This type of analysis has demonstrated reverse-mutation in two systems; the S locus (controlling response to streptomycin), and Lac (fermentation of lactose). Newcombe and Nyholm (to be published) and Demerec (1950) have studied streptomycin-sensitive reversions obtained by selection from streptomycin-dependent mutant strains. Most such reversions yield only sensitive prototrophs when crossed with type sensitive, and may thus be regarded as reverse-mutations. Demerec noted a variety of unrelated differences in some apparent reverse-

mutants, but it appears doubtful that these must all be attributed to changes at a single locus. E. Lederberg (1948) tested reversions of lactose-negative mutants to the type lactose-positive. All reversals which showed close phenotypic resemblance to type proved to be reverse-mutation as tested by crosses to type.

Suppressor and mimic mutations

In contrast to reversions demonstrably based upon reverse-mutation, a number of reversals have been found to depend upon mutation at loci other than that occupied by the original mutant gene. This can be shown by crossing the reversal to type and recovering the recombinant mutant phenotype. This result was obtained by Newcombe and Nyholm with one streptomycin-sensitive reversion and by Lederberg (1948) and E. Lederberg (1948) with a variety of reversals of fermentation mutants. As a rule, "suppressor" mutations are found most readily in stocks carrying alleles with an intrinsically low reverse-mutability.

In addition to "suppressors," which mimic the wild phenotype, many instances of mutations which mimic each other are known, posing the question of the number of loci involved. For example, Demerec and Fano (1945) described two mutations, (B/1 and B/1,5) in *E. coli* B, both of which manifested resistance to the virus T1, whereas only B/1,5 was resistant to T5. Without recombination test, it could not be shown whether these represent changes of similar or distinct genetic elements. Analogous mutants in K-12 can, however, be crossed with each other, permitting the identification of two distinct loci: V_{1a} and V_1 respectively.

Multiple alleles

Three series of multiple alleles have been recognized. Mutations from type (S^s) leading to resistance to (S^r) or to dependence (S^d) on streptomycin (Fig. 5) have been shown to be uni-local in unpublished work by Demerec and Zinder and by Newcombe and Nyholm. A series of Lac_1- (lactose-negative) mutants which can be differentiated by the rates at which they reverse-mutate to Lac+ have been shown to be allelic by E. Lederberg (1948), (Fig. 6). Finally, a third

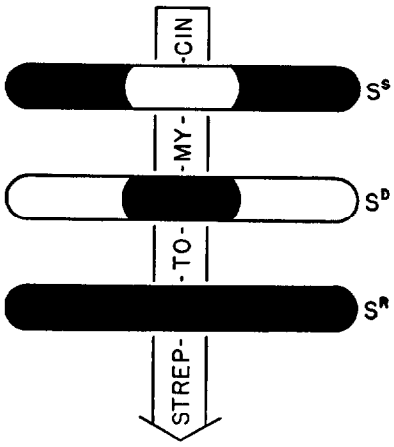

FIG. 5. Multiple alleles at the S locus, showing appearance of streptomycin-sensitive, -dependent, and -resistant forms when cross-streaked with streptomycin on agar medium.

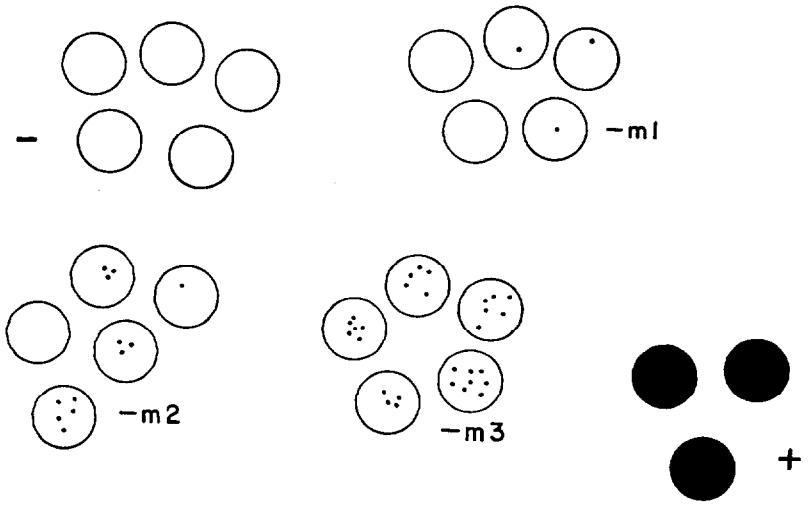

FIG. 6. Multiple alleles at the Lac_1 locus differentiable by their rates of reverse mutation. The dots represent "papillae" composed of reverted cells.

allele has been found at the V_1 locus. This allele V_1^p results in an intermediate degree of resistance to the viruses T1 and T5, (Fig. 7), but has been studied in detail only from a genetic viewpoint. The compound heterozygote, V_1^p/V_1^r shows the V_1^s phenotype, possibly placing this system in the category of "pseudo-alleles," although no crossovers between V_1^p and V_1^r have been found. (Parenthetically,

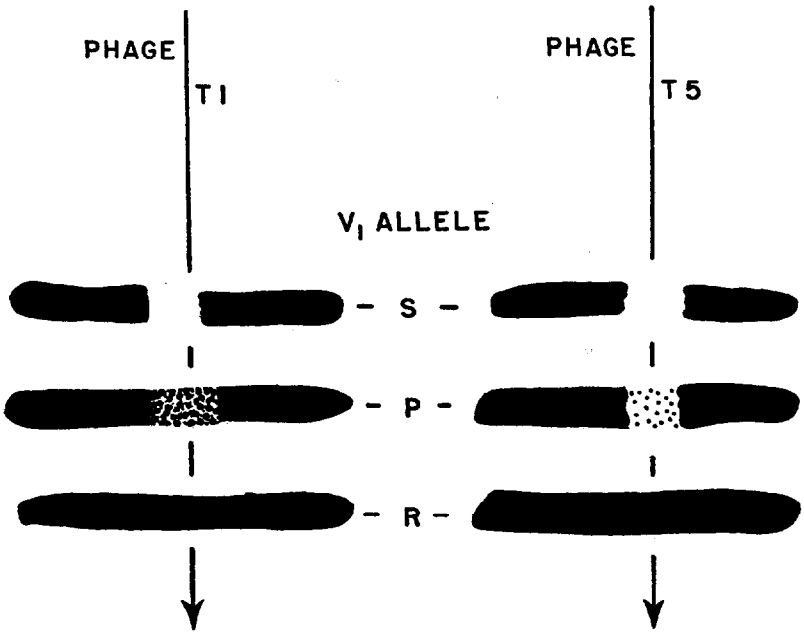

FIG. 7. Multiple alleles at the V_1 locus, showing modalities of response of sensitive, partially resistant and resistant cells when cross-streaked with viruses T1 and T5 on agar medium. The residual growth of V_1^p cells is due to incomplete lysis, not to the presence of a few resistant cells.

this "overdominant" phenotype was responsible for the first recognition of non-disjunctional types, Lederberg, 1949a). The structure of such genes cannot now be asserted by the bacterial geneticist any more than by the student of maize or fruit-flies, but at least the question can be put. However, microorganisms possibly provide material in which the physiological differentiation of genic components can be more readily studied.

GENETIC CONTROL OF ENZYME FORMATION

Dr. Sturtevant has suggested that the position effect is the most promising avenue of attack on the problem of gene function, and after having attempted some work on gene-enzyme relationships, I am inclined to agree with him.

As a preliminary approach, attention has been focussed upon a single "adaptive" enzyme, lactase, which can be readily assayed, and for which mutants are readily procured. It is, fortunately, very simple to isolate unlimited numbers of mutants in which this enzyme is affected, simply by plating irradiated populations on indicator medium containing lactose ("EMB-lactose"). Mutants lacking the enzyme can be found by inspection of colonies. Since one can easily score 500 or more colonies on a single plate at a glance, it has required no unusual effort during the last few years to examine perhaps a half-million colonies, from which about 300 mutations have been obtained. These have been tested for allelism by crossing them with each other. At least seven distinct genetic types were found, each giving wild type upon outcrossing with other mutants, but only lactose-negatives when crossed within the same type. It was concluded that mutation at any one of seven loci results in the substantial suppression of lactase formation in cultures exposed to lactose. In order to facilitate enzyme assays and extend their sensitivity, a lactose analogue, o-nitrophenyl-β-D-galactoside was employed. This compound, itself colorless, is split by lactase producing galactose and o-nitrophenol, whose yellow color permits spectrophotometric assay of the enzyme (Lederberg, 1950b). The use of this reagent also allows the investigation to be focussed upon a single enzyme, a simple hydrolytic glycosidase, rather than upon the complex of glycolytic or oxidative systems which are necessarily involved in manometric determinations of enzymatic activity.

More genes appear to be involved with this enzyme than I would have bargained for, but the next step is far more difficult, perhaps impossible: to analyze the mechanism of interference with lactase formation in the mutants, from which the function of the normal

allele might be inferred. Some of the mutants, Lac_2- and Lac_4-, show no promise in this direction, because as far as I have been able to determine they are absolutely and irreparably incapable of producing lactase and show no other phenotypic effect. Other mutants which show relative blocks may be more amenable.

For example, Lac_1- cells produce minimal amounts of lactase when grown on lactose, but substantial amounts on analogous substrates such as alkyl galactosides. (This results in the paradox that cells grown on a heterologous substrate are better adapted to lactose than those grown on lactose itself. Since "adaptation" is presumably a physicochemical rather than an entelechist process, such deviations are not surprising but suggest the need for revising "adaptive enzyme formation" in favor of a more general term connoting "enzyme formation under environmental influence.")

The Lac_3 locus offers even better possibilities, as it presents a clear-cut example of pleiotropic effects at the enzyme level. This mutant is defective in hexokinase (presumably), amylomaltase and lactase, resulting in an inability to split lactose and maltose or to glycolyze glucose (Lederberg, 1948; Doudoroff *et al*, 1949). In view of the complexity of effects the "single gene" nature of the mutation must be verified—the evidence for this seems to be as conclusive as could be obtained with any genetic analysis. The same phenotypic combination (glucose-, maltose-, lactose-negative) has been observed about twenty times among the three hundred odd lactose-negative mutants isolated. Attempts to find other types of glucose-negative mutants have led repeatedly to Lac_3- mutations. Some Lac_3- stocks throw reversions, selected on media containing any one of the three sugars as carbon sources, but in which the phenotypic effect is reversed *en bloc*. The reversions have been verified as reverse-mutations by crosses to wild type. Finally, the three effects are inherited as a unit in recombination experiments.

Two temperature sensitive Lac_3 types have been isolated (Lac_3t). Lac_3t shows the wild phenotype at 25°, but the typical Lac_3- at 40°C. The different effects show, however, distinct thresholds so that, for example, the phenotype at 36° is Glu−Mal−Lac+. The possibility of a common underlying mechanism with quantitatively

distinct thresholds for the three effects suggests itself immediately. If so, this might mean either a common "protogen" precursor for the enzymes, or some sort of inhibition. No enzyme-inhibitors have been found in crude preliminary experiments with mixed cultures, but current methods would in no case permit the detection of non-diffusible stimulants or inhibitors of enzyme formation, and there's the rub!

Ignorance is further amplified by a mutation, Cst+, which results in the so-called constitutive production of lactase. *E. coli* lactase is a classical example (Karström, 1930) of a bacterial adaptive enzyme, because cells grown on glucose show insignificant activity on lactose when tested manometrically, whereas lactose-grown cells glycolyze the disaccharide as rapidly as glucose. However, wild type cells grown on peptone, maltose, or other non-glucose substrates produce lactase in considerable, manometrically measurable amounts, and even glucose-grown cells show definite lactase activity (about one per cent of maximal), readily detected by colorimetric methods. A more adequate description of Cst+ would be, then, that it differs from type Cst− in the production of optimal levels of lactase on glucose and other non-lactose substrates.

The Cst+ mutant was first discovered in a rather interesting way. Neolactose, or altrose-galactoside, appeared to be inert to attack by wild type cells. I attempted, therefore, to select for a mutant with a lactase of altered specificity which could attack this analogue. The experiment was performed in the usual way, by inoculating large populations of cells into a medium with neolactose as carbon source. A "neolactose-positive" mutant was readily obtained in this way, and after purification was subjected to physiological study. Meanwhile, it was found that neolactose was split by type lactase, but that it was unable to elicit the formation of lactase by type cells (resembling the lactose-Lac_1-relationship). At first it was thought that the neolactose-positive mutants had an altered specificity of adaptive response so that neolactose would now be recognized as a stimulus. But it was soon found that optimum lactase (= neolactase) activity was developed whether or not a galactoside substrate was included in the growth medium. The necessary inference is that the adaptive

system (or an alternative one) has lost, rather than altered, its specificity of response. It should be noted that the production of maximal lactase activity by growth on glucose, and the absence of galactozymase activity in such cells argue against this mutant's responding to a sort of internal adaptation, viz., by intracellular formation of simple galactosides.

None of these mutations seem to affect the enzyme itself. In Lac_3t, it is not the enzymes but the conditions of enzyme formation which are temperature dependent. In Lac_1- and in Cst+ also, it is the specificity of enzyme formative responses, rather than the specificity of the enzyme which is altered. We are left with the rather unsubstantial conclusion that the complex process of substrate-dependent enzyme formation is subject to rather complex genetic control.

The debate of "true" versus "spurious" pleiotropism has been renewed and revised in recent years in the form of the one-to-one theory of gene enzyme relationships. The data presented here, as well as those of Landman (1950) on Neurospora lactase, and of Markert (1950) on Glomerella tryosinase, demand the rejection of this hypothesis in any form which is at the same time simple and general. We may conclude that observed enzymatic changes are often indirect consequences of genic alterations; this is not to imply that they are never direct. However, we lack critical evidence that any biochemically observable enzyme is a direct product of the action of a gene. The one fallacy of which we must be especially wary is the notion that an enzyme is produced by a gene, rather than by an integrated genotype or cell. Nor are we entitled to make a similar sweeping generalization concerning cell antigens, for a variety of secondary products must be expected to show antigenic specificities. Here, however, we have a number of examples where different alleles in heterozygotes behave independently in respect to the specificity of the cell products. This type of evidence of direct gene-to-product relationship is so far lacking for any microbial enzyme, but suggests one of the few workable criteria which can be used.

I am loath, however, to suggest a thorough rejection of the principle of monotropic gene action for the simple reason that there is no adequate alternative. Since any particular case of pleiotropism

may be spurious, the search for a unitary alteration of function is the only kind of experiment that we can carry out. I suspect, however, that the worker interested in problems of gene action soon branches out into an unlimited variety of non-genetic experiments. It may be wondered whether a geneticist might not function more effectively as a catalytic or mutagenic agent, attracting the attention of chemists, embryologists and others to the problems of protein synthesis and morphogenesis which are now almost insuperable obstacles to the completion of our analyses. Some of the fruits of such collaborations you have seen in the contributions immediately preceding.

RECOMBINATION IN THE NATURAL HISTORY OF BACTERIA

Further study of genetic recombination in bacteria should be both extensive and intensive. It is particularly pressing for us to learn some of the details of the morphological processes underlying recombination in *E. coli* K-12. The genetic analysis has convinced me that there probably is a fusion of elements, possibly of ordinary cells; less likely of specialized gametic forms. However, this conviction cannot be entirely secure without a microscopic observation of fusion accompanied by a demonstration of its genetic effect. K-12, although well suited for genetics, does not offer an encouraging prospect for the cytology of fusion owing to the infrequency of recombination and the apparent absence of any relevant distinctive forms. Several workers have, however, described aggregation or fusion processes in *Agrobacterium* (*Phytomonas*) *tumefaciens* (Braun and Elrod, 1946) which very strongly suggest a sexual process, and are possibly our best leads toward a cytological demonstration. As these authors point out, however, ". . . cytological studies alone will not suffice to clarify this question. It will be necessary to bring together in a single star different strains . . . and determine from this cross whether recombination of characters results." It is strongly hoped that such a program will be executed with this organism, and with others which show similar morphological hints of genetic exchange.

Tests for genetic recombination have been carried out to too

small an extent to permit any generalizations on the scope of its occurrence. After Tatum and I had made our first observations on K-12, we found that a similar approach had been unsuccessfully attempted previously (see Tatum and Lederberg, 1947, for references). Some years before, a brief note had appeared with the intriguing title "Mendelism among bacteria?" (Brown and Heffron, 1929.) The paper deals with the possible segregation of a single character difference in "*Bacillus lutzae*." However, the details are suggestive rather than convincing, and I have been unable to trace the culture.

The work with K-12 differs from previous investigations primarily in the application of a selective technique for the detection of recombinants. However, the particular choice of this strain appears to have been exceptionally fortunate, for a limited number of attempts to demonstrate recombination in a few other strains have been less successful. K-12 itself is not exceptional in any obvious way. According to Dr. C. E. Clifton of Stanford University (personal communication), it "was isolated by Dr. Blair in the fall of 1922 from the stools of a diphtheria convalescent and was identified at that time as an *E. coli* by the ordinary laboratory tests. It has been maintained in our stock culture collection since 1925 and is used in our laboratory as the typical coli culture."

Other typical coli cultures used in physiological and genetic studies which have been tested for recombination include the B strain, used in bacterial virus work, Davis' W strain, and the L strain used by Roepke and Ryan and their associates. Unfortunately, these tests have been quite negative, so that various genetic phenomena which have come up in these studies cannot be adequately analyzed. However, Cavalli and Heslot (1949) have discovered an *E. coli* strain which is moderately cross-fertile with K-12 (one success in about seven tests). In tests of forty *E. coli* cultures isolated from chickens, I have found two or three which cross with K-12, but at so low a rate as to make analysis difficult. The possibility of intra-fertile systems which do not cross with K-12 has not, however, been tested on a large scale. The extent to which K-12 or other sexual strains recombine genetic characters in their natural habitats is quite obscure. Unless

crossing can occur between rather widely separated, genetically differentiated clones, recombination may make a relatively small contribution to genetic variability since in experimental material it occurs rather infrequently compared to clonal propagation. It must take place between stocks differing in at least two characters to have any effect at all in the production of new genotypes. However, in the laboratory, recombinants dually resistant to streptomycin and sodium azide (Az^rS^r) occur about one hundred times as frequently in mixed single-resistant cultures ($Az^rS^s + Az^sS^r$) as do mutations of the parental stocks to the same end genotype (Lederberg, 1950a). The potential significance of this finding for chemotherapy needs no elaboration.

Other bacteria have been scarcely studied at all from this viewpoint, and no publications can be cited as yet. However, this kind of investigation is being pursued in several laboratories, and, we may anticipate, not entirely fruitlessly.

"INFECTIVE" HEREDITARY TRANSMISSION

Two of the preceding papers in this book have considered at length the role of extrachromosomal (so-called "cytoplasmic") factors not only in heredity of microorganisms, but in heredity and development of higher forms. What contribution can a bacteriologist make to this discussion? It will be apparent at the outset that, for the most part, we can ill discuss extrachromosomal inheritance without first building a clear picture of the chromosomal mechanisms, if any. For reasons stated above, this distinction can rarely be made. The bacteria are notable, however, for examples of "infective" heredity, that is to say the transmission of genetic elements despite cellular discontinuity. The most outstanding and the best investigated of these examples is the pneumococcus transformation (McCarty, 1946). Whether or not the pneumococcus also possesses a "chromosomal" or nuclear genotype (and I see no reason to doubt that it does), the analogy which has been drawn between the transforming agents of pneumococcus and cytoplasmic factors in other organisms remains an instructive one (Sonneborn, 1943). I would like to suggest that a rather simple, unitary picture of extranuclear mechanisms can be

developed if we include in our discussion of "infective heredity" agents responsible for "infective pathology" such as intracellular viruses (Altenburg, 1946).

We have already heard many comments upon the difficulty of deciding upon the evolutionary origin or contemporary taxonomy of deleterious parasitic viruses at one extreme, and integrated cytoplasmic genes like plastids, at the other. Within this interval, we find a host of transition forms; kappa, lysogenic bacteriophages, genoids, tumor-viroids, male-sterility factors, Ephrussi's yeast granules, etc. These differ in several parameters, particularly the frequency with which spontaneous "disinfection" occurs, current experimental success in achieving artificial infection, and the pathology of the infection, that is, the phenotype corresponding to it. None of these differences is really fundamental from a genetic point of view, because the cell-virus complex is at least theoretically capable of adaptive evolution as a consequence of natural selection and mutation in either the virus or the cell component, or both. The objection has been voiced that this viewpoint is an attempt to relegate plasmagenes to pathology. I rather think that it may broaden our genetic insights if we consider the likenesses as well as the dissimilarities between pathogenic viruses and plasmagenes. For example, we would not have learned how to "cure" green plants of their chloroplasts (Provasoli, Hutner and Schatz, 1948) if it were not for more mundane chemotherapeutic investigations.

Extra-chromosomal agents which can be transferred outside the cell obviously provide the most suitable material for experimental study of their composition, numbers, morphological structure and so on. From this point of view, the lysogenic viruses carried by many bacteria provide the best material, especially as infection with such a virus is formally indistinguishable from events such as pneumococcus transformations. (For an amplification of this viewpoint, and further literature references see Lederberg, 1949b, especially pp. 17-19.)

Perhaps less profitably, a further generalization may be erected. I wonder whether we cannot split the organism into smaller functional units than the cell, depending upon their integration into

genetically continuous aggregates. For example, the yeast cell would consist of the interaction of its nuclear genotype, its cytoplasmic-granule genotype, and so forth. Our examples of cytoplasmic determination would then correspond to a series of interactions of different genotypes with varying degrees of mutal dependence. In a sense this is equivalent to conferring quasi-organismic status upon each of the different components. The advantage (or drawback) of this unifying view is that it comprehends a continuous spectrum of such genotypic interactions ranging from Ephrussi's granules, lysogenic viruses, and facultative intracellular symbiosis eventually to the least tangible ranges of genotypic (that is, interorganismal) interaction in, for example, human social relations. At each level of interaction pathological deviations can be found, ranging from sick plastids and malignant tumors (on Darlington's theory) to human serfdom.

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[1] These references are not intended as a comprehensive bibliography of bacterial genetics. For extensive literature lists see Lederberg, 1949b, and: Luria, S. E., 1947, Recent advances in bacterial genetics. Bact. Revs. 11:1-40; Braun, W., 1947, Bacterial dissociation. Bact. Revs. 11:75-114; Lederberg, J., 1948. Problems in microbial genetics. Heredity 2:145-198; and Tatum, E. L. and Perkins, D. D., 1950. Genetics of microorganisms. Ann. Rev. Microbiology 4:129-150. Readers are also referred to a forthcoming reprinting of selected articles on microbial genetics to be issued by the University of Wisconsin Press.

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